IN-SITU SINGLE DNA MANIPULATION WITH f20nm ELECTRON-BEAM-DEPOSITED PROBE

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Abstract-**This paper proposes a novel DNA-sequencing-preprocessing that can selectively extract a specific part of a single molecular DNA fiber by in-situ manipulation. The in-situ manipulation extends DNA fibers on a glass substrate, cuts into several DNA fragments, and isolates a specific DNA fragment. In this method full-time observation of the manipulation is provided for the purpose of keeping the information about where the isolated DNA fragment used to be located in the original DNA fiber. A hook-shaped f 20 nm probe was fabricated by Electron-Beam-Deposition for isolating the DNA fragment. With this probe, the DNA fragment, extended on the surface of water-repellent with its end anchored by Au colloid, was hooked and lifted up from the glass substrate; water-repellent has an effect of preventing adsorption between the DNA fragment and the glass substrate. Finally, the specific DNA fragment was successfully isolated onto a new clean glass substrate.**

I. INTRODUCTION

Current sequencing method, Sanger method, can analyze up to about 1000 bp (base pairs) at a time. Therefore, YAC (Yeast Artificial Chromosome) clones has to be cut into fragments under 1000 bp by restriction enzymes, and each fragment has to be isolated by electrophoresis or plasmid colonies [1,2]. Because these processes is carried out in solvent, we lose important information about where the original fragments used to be located; another laborious efforts are devoted to reorganize them by computing [3]. Thus, more efficient preprocessing is greatly desired.

Previously, we proposed a alternative method for preprocessing of genome analysis based on the mechanical manipulating techniques [4], called "in-situ manipulation," in which all operations were done on a glass substrate under fluorescent microscope observation. This full-time observation of a single molecular DNA fiber allows us not to lose information of the original location of the fragment in the whole genome. As shown in Fig. 1, this method consists of four processes: i) extending DNA fibers on the glass substrate; ii) cutting the single DNA fiber mechanically or with restriction enzymes; iii) isolating the fragment from the glass substrate; and iv) amplifying the fragment by PCR (polymerase chain reaction). Out of these processes, this paper present extending, cutting, and isolating; the process of amplifying will be our future work.

Previously several preprocessing methods based on manipulation have been proposed. Washizu et al. extended and aligned DNA fibers on a gelatin layer, cut the DNA fibers into fragments with an AFM cantilever, and isolated the fragments together with the gelatin layer [5]. They aligned the DNA fibers by anchoring one ends of the DNA fibers and extending with high frequency voltage. However, this method can't cut the specific part of the DNA fibers because the DNA fibers couldn't be aligned with their heads and tails in the same direction. Mizuno et al. manipulated a single molecular DNA by trapping a biotin-labeled DNA with a avidin-labeled 1 μm bead controlled by a laser tweezer [6]. However, the 1 μm bead is too large for manipulating a 1000 bp DNA for base-sequencing. Another problem is contamination of unexpected DNA fragments; as the manipulation is conducted in solvent, unexpected DNA fragments in the solvent may contaminate the isolated fragment. Ikai et al. isolated a single molecular DNA using an AFM cantilever as a probe [7]. They trapped a single molecular DNA fiber by adsorbing to the probe. However, they had to control the pH of the solvent to reduce adsorbability between the DNA fibers and the substrate. But the probability of this method was 10 % at most. In addition, this method also has the problem of contamination; unexpected DNA fibers may stick to the probe. These previous research shows following major problems of DNA manipulation remain to bo solved: a) contamination of the unexpected DNA fragments, that is, DNA fibers moving around in the solvent may stick to the probe and cause contamination; b) adsorbability between the DNA fibers and the substrate, which obstruct lifting-up the DNA fragment from the substrate; and c) demand for a thinner probe, probe for manipulation must be thin enough to lift-up DNA fragment mechanically, that is, tip diameter of probe should be smaller than 300 nm (length of 1000 bp DNA fiber).

In this paper we present a new in-situ manipulation method to overcome these problems. We extend DNA fibers on the surface of fluid water-repellent by anchoring the ends of the DNA fibers by Au colloid. This extension method allows us to isolate the DNA fragment out of solvent, which clears the problem a); unexpected DNA fragments sticking to the probe and contaminating the isolated DNA fragment. Using water-repellent also reduces adsorbability between the DNA fibers and the substrate and clears the problem b).

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Abstract

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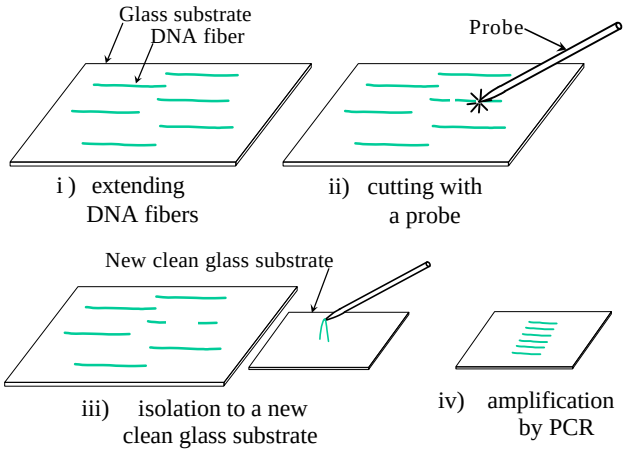


Fig. 1. Schematics of in-situ manipulation for preprocessing of genome analysis.

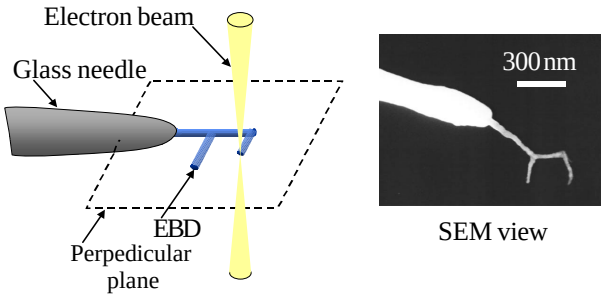


Fig. 2. Schematic and SEM view of EBD. A ϕ 20 nm wire-frame is grown in a plane.

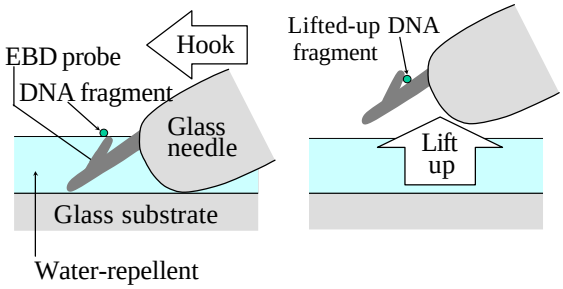


Fig. 3. Schematic of lifting-up of a DNA fragment. The EBD probe hooks the DNA fragment floating on the surface of water-repellent and lifted up the fragment with a hook-shaped EBD probe.

We fabricate a nanometer-sized probe with Electron-Beam-Deposition to clear the problem c). EBD is carbon-deposition of residue-gas-molecules dissociated by a electron beam of SEM [8,9]. As shown in Fig. 2, by scanning the focused electron beam from a base EBD can shape a ϕ 20 nm-wire-frame as is desired, which is thin enough to isolate a 1000 bp DNA fragment. As shown in Fig. 3, a hook-shaped EBD probe can lift-up and isolate the DNA fragment floating on the surface of the fluid water-repellent layer. Through experiments, we show feasibility of water-repellent and hook-shaped EBD probe to remove the problems and isolate a DNA fragment as a sample for base sequencing.

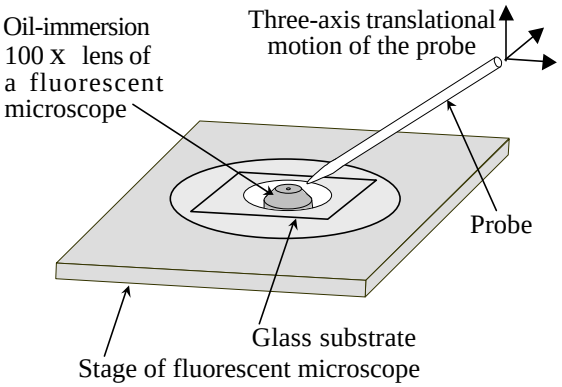


Fig. 4. Illustration of Experimental setup of in-situ manipulation. Probe is manipulated by a three-axis water hydraulic micromanipulater. Observation was provided by a fluorescent microscope.

II. Experimental

Fig. 4 shows the experimental setup for in-situ manipulation. We manipulated DNA fibers on the stage of a inverted fluorescent microscope, Olympus IX-70, equipped with a 100 x oil-immersion lens, Uplan Apo 100 x oil Iris. The probe was manipulated by a three-axis water hydraulic micro-manipulator, Narishige MHW-3. DNA fibers were dyed with YO-PRO-1 iodide. Fluorescent images were obtained with a high sensitivity CCD camera, Olympus HCC-600, and recorded by a video recorder.

A. Extension

To evaluate the effect of water-repellent, we prepared three substrates, 40 mm x 50 mm x 0.17 mm glass plates, as follows.

a) a substrate with no treatment.
b) a substrate coated with water-repellent.
c) a substrate coated with water-repellent and Au colloid on the surface of the water-repellent layer. Au colloid was expected to act as anchors for DNA fibers to extend on water-repellent surface.

For preparing substrate b) and c), 20 µl, 20 % diluted water-repellent solution in PFC, Optool Daikin industries, was pipetted on 100 mm^2 area of the substrates. Then for substrate c), 10 µl, 5 ppm solution of ϕ 0.5 nm-1nm Au colloid in purified water was pipetted on the substrate, and then made to run over the surface of water-repellent by tilting the substrate to a vertical posture.

Then we let the DNA solution flow on the prepared three substrates to extend DNA fibers on them; mixed solution of 10 µl, 5 ng / µg lambda DNA solution in purified water and 1 µl, 1 µM YO-PRO-1 iodide was pipetted on the three substrate, and then was made to run over the surface of the substrate by tilting the substrate in a vertical posture.

To evaluate the adsorbabilty between the DNA fiber and the substrate, we pushed extended fibers with ϕ 200nm glass needle made by a micro-pipette puller, Narishige PN-30, and

observed the motion of the DNA fiber.

B. EBD Probe Fabrication

We made a EBD probe for isolation of a DNA fragment. First, we prepared a ϕ 200nm glass needle for a base of the EBD probe using a micro-pipette puller, and then coated the needle with a 10 nm gold layer with sputtering-machine, SANYU DENSHI SC-701, preventing the problem of charging-up by electron beam. Then we grew a EBD probe in the chamber of SEM, HITACHI S-4160. We fabricated thin needle-structure at the tip of the glass needle to insert under the DNA fiber floating on the substrate, and two hooks on the upper part of the probe.

C. Cutting and Isolation

We cut an extended DNA fiber on the substrate c) into three fragment by manipulating a ϕ 200 nm glass needle.

Then, to evaluate the effect of the EBD probe, we conducted isolation of a specific fragment with two probes; the EBD probe fabricated above and a ϕ 200 nm glass needle as a reference. We lifted down the probe tip until it touched the substrate, moved it horizontally toward the DNA fiber until it hooked the DNA fiber, and then lifted it upward. After lifting up the DNA fragment to the air, we transported the fragment onto a new clean glass substrate and confirmed there was no other DNA fiber on the new substrate.

III. Results and Discussions

A. Extension

Fig. 5 shows the fluorescent images of DNA fibers extended on the substrate a), with no treatment, and substrate c), coated with water-repellent and Au colloid on the surface. On the other hand, DNA fibers were not extended on the substrate b), coated with water-repellent without Au colloid. This result testify the effect of water-repellent and Au colloid on the surface. Water-repellent isn't thought to adsorb DNA fibers on its surface. Conceivably particles of Au colloid, spread on the surface of water-repellent, act as anchors for DNA fibers. The maximum length of the DNA fibers shown in Fig. 5 is about 15 μm, proper length for lambda DNA containing 48,502 bp, which shows the DNA fibers are single molecular.

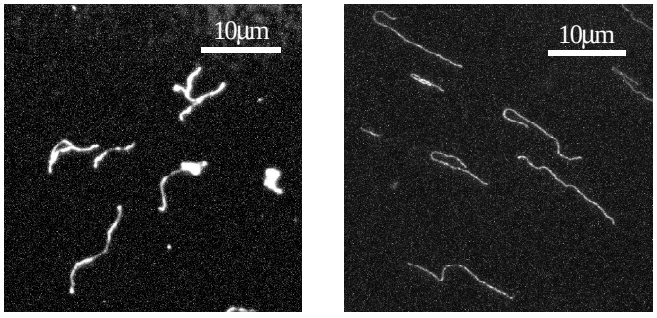
DNA fiber on the substrate a) wouldn't move by pushing with the ϕ 200 nm glass needle. On the other hand, DNA fibers on the substrate c) was easily moved as shown in Fig. 7. This results shows that water-repellent is effective for reducing adsorbability and moving the DNA fibers on the substrate.

B. EBD Probe Fabrication

Fig. 6 shows the SEM image of the EBD probe for isolation. The ϕ 20 nm wire-frame was successfully fabricated as designed.

C. Cutting and Isolation

With the ϕ 200 nm glass needle, the probe for reference, we couldn't lift up a DNA fiber on the surface of water-repellent.



1) Substrate a) , with no treatment.

2) Substrate c) , coated with water-repellent and Au colloid

Fig. 5 Fluorescent microscopic views of lambda DNA fibers extended on the substrate. The fibers of image 2) is about 15μm long. which is the length of lambda DNA (48,502bp).

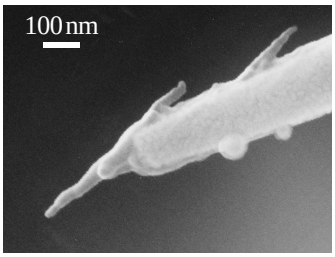
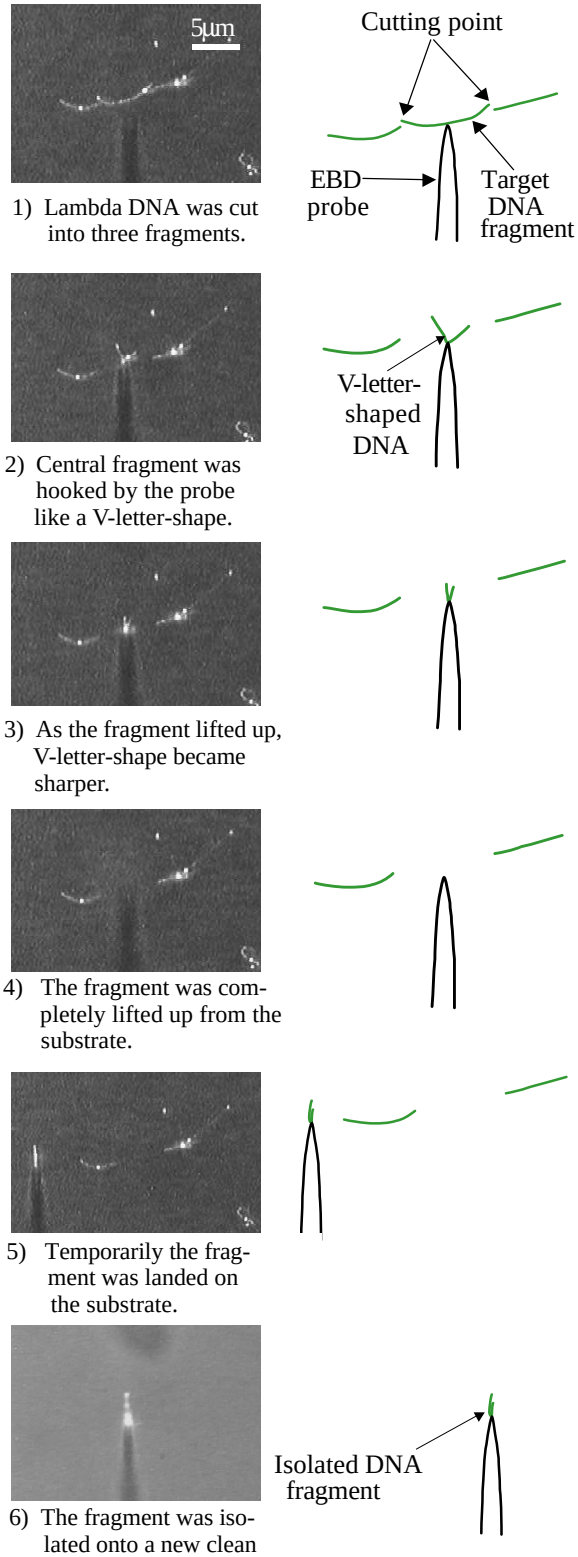


Fig. 6 SEM view of the hook-shaped EBD probe. There are two hook-shaped structures on the probe. Its tip was made thin to hook the DNA fiber floating on water-repellent.

On the other hand we were successful in isolation with the EBD probe shown in Fig. 6. The fluorescent images of isolation with the EBD probe are shown in Fig. 7; 1) an extended lambda DNA fiber had already been cut into three fragments by the glass needle. The EBD probe for isolation was approached to the center DNA fragment; 2) and 3) the center DNA fragment, hooked and lifted up by the probe, observed like a V-letter-shape; 4) the fragment was completely lifted up from the glass substrate and out of the focus of the microscope; 5) the lifted DNA fragment was temporarily landed onto another part of the same glass substrate; 6) the fragment was transported and put down onto a new clean glass substrate. Only the isolated DNA fragment was observed on the new substrate, which shows there was no contamination of unexpected DNA fragments. This result proved the hook-shaped EBD probe to be useful for lifting up the DNA fragment floating on the surface of the water-repellent.

However, water-repellent may coat the isolated fragment through our method and prevent the reagent of next amplifying process. Probably, there is a necessity of washing away water-repellent from the isolated fragment with surface-active agent.

We are aiming at manipulating YAC clones in the final stage of our study, for current genome analysis is based on sequencing each YAC clone prepared from the genome library [10]. We



1) Lambda DNA was cut into three fragments.

2) Central fragment was hooked by the probe like a V-letter-shape.

3) As the fragment lifted up, V-letter-shape became sharper.

4) The fragment was completely lifted up from the substrate.

5) Temporarily the fragment was landed on the substrate.

6) The fragment was isolated onto a new clean glass substrate.

Fig.7 Fluorescent microscopic view and illustration of isolating a DNA fragment. visible light is radiated together with excitation light to observe the probe.

are going to develop the next process of amplification of the isolated DNA fragment by PCR, which may accelerate genome analysis.

IV. Conclusion

We proposed a novel DNA-sequencing-preprocessing that can selectively extract a specific part of a single DNA fiber by in-situ manipulation.

We proposed and verified the feasibility of using water-repellent and hook-shaped EBD probe for isolating a specific single molecular DNA fragment. First, we fabricated a hook-shaped ϕ 20 nm probe by Electron-Beam-Deposition for isolating the DNA fragment. With this probe, we hooked and lifted up the DNA fragment extended on the surface of water-repellent by anchoring its end by Au colloid; water-repellent was useful for preventing adsorption between the DNA fragment and the glass substrate. Finally, we transported the DNA fragment onto a new clean glass substrate. No contamination of unexpected DNA fragments was observed on the new substrate, which shows we were successful in isolation.

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